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Original Article

Superantigen involvement and susceptibility factors in Kawasaki disease: profiles of TCR Vβ2+ T cells and *HLA-DRB1*, *TNF-α* and *ITPKC* genes among Filipino patients

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Abstract: Superantigens and genetic factors may play roles in the etiology and susceptibility to Kawasaki disease (KD). To investigate these roles, percentages of TCR-Vβ2+ T cells were compared by flow cytometry using anti-Vβ2 monoclonal antibodies and genotyping was done on *HLA-DRB1* exon 2, the -308 site of the *TNF-α* promoter region, and *ITPKC* SNP rs28493229 by polymerase chain reaction followed by direct sequencing. There were higher percentages of Vβ2+ T-cells in KD patients (9.5 ± 2.15%) compared to healthy controls (7.25 ± 1.48%) ($P<0.05$, Student's t-test, n=6-8/group). However, no polymorphisms were observed in exon 2 of *HLA-DRB1* and in the -308 region of the *TNF-α* promoter. The *ITPKC* SNP rs28493229 G/C polymorphism was observed in 1 KD patient and 4 healthy controls. This study suggests that KD etiology may be associated with a superantigen and that *HLA-DRB1* exon2, *TNF-α* -308 region and *ITPKC* SNP rs28493229 may not be associated with KD. This is the first study investigating Vβ2+ T cells and candidate genes involvement among Filipino KD patients.

Keywords: Superantigens, HLA-DRB1, TNF-α, ITPKC, Kawasaki disease

Introduction

Kawasaki disease (KD) is an acute potentially fatal multisystem vasculitis most commonly occurring in children less than 5 years of age [1]. It is considered as the most common cause of acquired heart disease in children that may lead to the development of coronary artery abnormalities when left untreated [1].

The causative agent of KD, which is believed to be infectious in nature, has not been identified. Studies have shown that KD may be caused by a superantigen (SAg), which causes a selective increase of T cells expressing particular Vβ gene segments [2-8]. Moreover, emerging lines of evidence suggest that genetic factors are involved in the susceptibility and outcome of KD. Asian children, especially those of Japanese, Chinese, and Korean descent, have the highest incidence of KD, although all racial groups are affected, strongly suggesting a genetic predisposition for KD [9]. The most probable candidate genes for susceptibility are those involved in immune reactions that characterize KD, such as *HLA-DRB1*, tumor necrosis factor-alpha (*TNF-α*) and inositol1,4,5-triphosphate(IP3) kinase (*ITPKC)*. SAgs bind not only with the Vβ region of the TCR but also with major histocompatibility (MHC) class II molecules expressed on antigen presenting cells (APCs) [10]. An elevation of TNF-α during the acute phase of KD has been implicated in the pathogenesis of vasculitis in KD [11-15]. Its levels are highest in children who develop coronary artery aneurysm (CAA) [15-17]. Polymorphisms in the promoter region of *TNF-α* have been associated with coronary artery disease (CAD) [18], coronary heart disease (CHD) [19], rheumatoid arthritis (RA) [20-22], rheumatic fever [23], and acute coronary syndrome (ACS) [24]. Some KD patients are known to develop CAD [25]. In a genome-wide study (GWAS) of KD, Onouchi et al identified a functional SNP (itpkc_3 G/C [rs28493229]) within

intron 1 of the *ITPKC* gene to be associated with KD [26].

Studies such as these have not been done among Filipinos. We investigated if there was a significant increase in the percentage of TCR-Vβ2+ T cells in KD patients compared to healthy controls to suggest SAg involvement, and if polymorphisms in *HLA DRB1* exon 2 (where most polymorphisms occur), the -308 polymorphism hotspot in the *TNF-α* promoter region and the functional SNP [rs28493229] in intron 1 of *ITPKC* were associated with susceptibility to KD.

Methodology

Two groups of patients were included in this study. The first group was composed of young children who were diagnosed clinically with KD with the following criteria: fever for at least 5 days, and four of the following five signs, (1) bilateral conjunctival injection without exudates; (2) changes in the oral mucosa, such as erythema and cracking lips, erythema of the pharynx, strawberry tongue; (3) changes in extremities, such as redness and swelling in the acute phase, periungual desquamation in the subacute phase; (4) polymorphous exanthema, and (5) cervical lymphadenopathy, (≥1.5 cm in diameter), usually unilateral. No other disease processes could explain the illness. Atypical cases were excluded.

These patients were referred to physicians at the Philippine Children's Medical Center, Quezon City, and the Far Eastern University-Dr Nicanor Reyes Medical Foundation Medical Center, Quezon City.

The second group was composed of healthy controls with ages ranging from 18 to 27 years, who were free of febrile disease for at least 4 weeks before examination and who were not under the influence of any treatment. Because it was difficult to obtain parental consent for the collection of blood samples from healthy young children, an older age group was used as healthy control. Van den Beemd *et al* showed that there is no difference in the Vβ2 usage between age groups 0-15 years and 16-30 years, with both groups having a mean value of 7.9% [27].

Informed consent was obtained from the parents/guardians of the patients or from the healthy controls who have reached the age of majority at the time of sample collection. The research protocol was reviewed and approved by the Institutional Ethics Review Committee of the Far Eastern University-Dr. Nicanor Reyes Medical Foundation with approval number BS-F-005-2011.

Approximately 2 to 3 ml of peripheral blood were drawn using EDTA as anticoagulant within 10 days of the onset of fever from patients suspected of having KD and before the administration of IVIG, and anytime from healthy controls.

To determine the percentage of Vβ2+ T cells, fluorescent activated cell sorting (FACS) was done to count CD3(+) T-cells by reacting them with FITC-conjugated anti-CD3 antibody (Clone IM1281, Immunotech, Marseille, France). Out of these CD3(+) T-lymphocytes, the percentages of those bearing TCR Vβ2 were determined using phycoerythrin-conjugated monoclonal antibody (Clone MPB2D5, Immunotech, Marseille, France) against TCR Vβ2.

To determine the polymorphisms in exon2 of *HLA-DRB1*, -308 site of the *TNF-α* promoter region and the *ITPKC* 1 SNP [rs28493229], genomic DNA was extracted from the blood samples using Genomic DNA Mini Kit Frozen Blood Protocol (Geneaid Biotech Ltd., Sijhih City, Taiwan). In this part of the study, 11 patients were added to the previous 6, constituting a total of 17 KD patients and 18 were added to the 8 with a total of 26 healthy controls. These genomic DNA extracts were used to amplify *HLA-DRB1* exon 2, the *TNF-α* promoter containing the -308 region and the *ITPKC* intron 1 encompassing the functional SNP [rs28493229]. The sets of gene-specific primers used are shown on **Table 1**.

Gene amplification via polymerase chain reaction was performed using Promega Master Mix (Promega Corp, Wisconsin, USA) with the following PCR conditions for *HLA-DRB1*: initial denaturation at 95°C for 3 minutes and 35 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 1.5 min, and a final extension step for 3 min at 72°C; for *TNF-α* promoter region: initial denaturation at 95°C for 3 minutes and 35 cycles of 95°C for 30 s, 55.3°C for 30 s, and 72°C for 1.5 min and a final extension step for 3 min at 72°C; and for *ITPKC* intron 1[rs28493229]: initial denaturation at 95°C for

Table 1. Primers used in the amplification of the gene regions

Gene	Primer sequences	Amplicon size
HLA-DRB1 exon 2		
Forward primer	5'-cagcacgtttcctgtggcag-3'	261 bp
Reverse primer	5'-ctgtgaagctctccacaaccc -3'	
TNF-α promoter (-308)		
Forward primer	5'-gaaggaaacagaccacagacc -3'	187 bp
Reverse primer	5'- ggggacacacaagcatca -3'	
ITPKC intron 1 [rs28493229]		
Forward primer	5'-ctggcactggtggtttccaaat-3'	500 bp
Reverse primer	5'-aagaggttcccggagatgaaattg-3'	

Table 2. TCR Vβ2+ T-cell expression in KD patients and healthy controls

	Day of illness blood was extracted	TCR V-beta2+ T cell/ Total CD3+ T cell (%)
KD Patients		
K01	8	10.3
K03	6	8.6
K04	8	10.9
K05	6	12.4
K06	8	8.5
K07	10	6.3
Healthy controls		
C01		8.8
C02		6.6
C03		5.1
C04		7.2
C05		9.7
C06		6.5
C7		6.3
C08		7.8

3 minutes and 35 cycles of 95°C for 30 s, 55.0°C for 30 s, and 72°C for 1.5 min and a final extension step for 3 min at 72°C. The PCR products were sent to FirstBASE Laboratories Sdn Bhd (Malaysia) for direct sequencing of forward and reverse strands using the same gene-specific primers.

Student t-test was used to analyze the difference in the percentages of Vβ2+ T cells between KD samples and those of healthy controls. A p-value of <0.05 is considered significant. Genotype and allele frequencies of SNP rs28493229 were determined by direct counting. Chi square test using Fisher's exact test was used to determine significance. Odds ratios and 95% confidence intervals were also calculated.

Results

The Vβ2+ T-cell expression in 6 KD patients and 8 healthy controls are shown in **Table 1**. The KD patients had Vβ2+ T cell percentages that ranged from 6.3% to 12.4% while that of the healthy controls ranged from 5.1% to 9.7% (**Table 2**). There were higher percentages of Vβ2+ T-cells in KD patients (9.5 ± 2.15%) compared to healthy controls (7.25 ± 1.48%) ($P<0.05$, Student's t-test, n=6-8/group) (**Table 3**).

No polymorphisms were observed in the DNA sequences of *HLA-DRB1* in both the KD and healthy controls. All KD sequences were those of DRB1*15:01:01:01 and healthy controls were also those of DRB1*15:01:01:01. Likewise, no polymorphisms were detected at the -308 position of the *TNF-α* promoter. We found that all KD patients and all controls had *GG* alleles at this position.

There were no differences in the genotype (OR=0.34, 95% CI=0.035-3.374, p=0.33) or allele (OR=0.36, 95% CI=0.39-3.401, p=0.34) frequencies of *ITPKC* SNP rs28493229 between controls and children with KD (**Table 4**). Only one out of 17 (5.9%) KD patients had G/C genotype and 4 out of 26 (15.4%) controls had the same genotype. Similarly, the *C* allele was found in only one KD (2.9%) patient and in 4 (7.7%) of the controls.

Discussion

Kawasaki disease is postulated to be caused by a SAg, which may be produced by a ubiquitous infectious agent. The infectious origin of KD has been hypothesized because of the nature of its clinical manifestations and epidemiological characteristics [1]. The SAg theory has been the subject of several investigations with conflicting results. The hallmark of a disease caused by a SAg-producing organism is the selective expansion of T cells bearing a par-

Table 3. Student's t-test analysis of V-beta-2+ T cells in KD patients

	Status	*n*	mean	SD	*p*-value
V-beta-2+ T cell/Total CD3+ T cell (%)	KD	6	9.50	2.151	0.038
	Control	8	7.25	1.475	

Table 4. Polymorphism of rs28493229 of the *ITPKC* gene in KD patients

	KD, *n*=17 (%)	Control, *n*=26 (%)	OR	95% CI	X^2 Fisher's Exact test p-value
Genotype					
C/G	1 (20.0)	4 (80.0)	0.34	0.035 – 3.374	0.33
G/G	16 (42.1)	22 (57.9)			
Allele					
C	1 (20.0)	4 (80.0)	0.36	0.39 – 3.401	0.34
G	33 (40.7)	48 (59.3)			

ticular β-chain variable gene segments [28, 29]. One group of investigators has shown positive evidence of SAg etiology showing either a selective expansion of Vβ2- or V8-bearing T cells or the isolation of SAg-producing bacteria from the patients [3-5, 8, 30]. Another group, however, failed to demonstrate the above findings, neither were they able to find a significant difference in the presence of SAg-producing bacterial isolates among KD patients and control, nor did they see a pattern of increased Vβ family of T cells [31-33].

To investigate a SAg involvement in KD among Filipino patients, we performed fluorescence activated cell sorting (FACS) and found that KD patients have higher Vβ2+Tcells compared to healthy controls, similar to the observation of Reichardt et al of elevated percentages of Vβ2.1+ T cells in 7 confirmed KD patients compared to healthy controls [6]. Brogan et al demonstrated that 13 of 16 (81%) of their KD patients had either Vβ skewing and/or Vβ restricted activation involving Vβ2.1 and Vβ5.1 [34]. This is the first study that shows Vβ2 profiles in Filipino KD patients.

Genetic factors may also be involved in the susceptibility to KD. A few studies have been conducted on the role of MHC class II in susceptibility in KD. Yoshioka et al previously reported that the frequencies of the DRB1*04051, *0406, and *0901 were high, whereas that of the DRB1*1101 was low among patients with KD as compared with the healthy adults [8]. We attempted to implicate polymorphisms in two candidate genes, namely, *HLA-DRB1* and *TNF-α*, with KD, both of which yielded negative results. The *HLA-DRB1* allele of all our 17 KD patients was HLA-DRB1*15:01:01:01 and the same allele form was seen in all the 26 healthy controls. Similarly, Huang et al reported that the distribution of HLA-DRB1 allele families and alleles in children with KD did not differ from that in healthy controls in Taiwan [35]. Because the *TNF-α* gene locus lies within the HLA complex, we investigated its polymorphism specifically at the -308 position of its promoter with the aim of determining whether this region is influenced by the polymorphism in *HLA-DRB1*. TNF-α is involved in infectious and immunoinflammatory diseases such as KD. Different individuals may have different capacities for TNF-α production. TNF-α levels are elevated in the majority of children during the acute phase of KD [15, 16] and are highest in children who develop coronary artery aneurysm [15-17]. Most studies were concentrated in the promoter area of the *TNF-α* gene [36]. In our study, no polymorphism was observed at the -308 locus among the KD patients and healthy controls. These findings suggest that susceptibility to KD and coronary artery lesions may not be associated with the HLA-DRB1 and TNF-α gene.

We did not find any significant difference in the genotype or allele frequencies of *ITPKC* SNP rs28493229 between controls and children with KD. Only one out of 17 (5.9%) KD patients had C/G genotype and 4 out of 26 (15.4%) had the same genotype. There was higher percentage (7.7%) of controls with C allele than those of the KD patients (2.9%). This is contrary to the findings of Onouchi et al [37] in their study among Japan and US patients, where C allele was present in greater frequency in KD patients than in healthy controls.

In conclusion, we show that there are Filipino patients who have elevated Vβ2+ T cells compared to healthy controls, suggesting a possible involvement of a SAg in the etiology of KD. However, there was no difference in the polymorphisms found in *HLA-DRB1*, *TNF-α* and *ITPKC* genes between the two groups. Further studies on TCR-Vβ2+ T-cells and genetic polymorphisms relating to susceptibility involving a larger cohort of Filipino patients would help elucidate the problem of KD etiology, pathogenesis, and susceptibility in the Philippines.

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